Identification and Profiling of Microbial Community from Industrial Sludge

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Abstract

This study aims to identify microbial communities and their taxonomical profiling basis of order, species, genus, family, and class at the level in the sludge of pulp and paper industry. Studies showed dominant phyla in 16S rDNA Illumina Miseq analysis inside sludge were *Anaerolinea*, *Pseudomonas*, *Clostridia*, *Bacteroidia*, *Gammaproteobacteria*, *Spirochetia*, *Deltaproteobacteria*, *Spirochaetaceae*, *Prolixibacteraceae* and some unknown microbial strains are also dominant. The results of metabarcoding of the V3-V4 16S rRNA regions acquired from paired-end Illumina MiSeq sequencing were used to analyze bacterial communities and structure. Microorganisms can produce a vast variety of secondary metabolites, all of which are playing a crucial in biogeochemical cycle processes. The present work demonstrates the potential approach to sludge treatment in the open environment via the naturally adapted microorganism, which could be an essential addition to the disposal site. In summary, these investigations indicate that the indigenous microbial community is an acceptable bioresource for remediation or detoxification following secondary treatment. This research aims at understanding the structure of microbial communities and their taxonomic diversity (%) in highly contaminated sludge to perform in-situ bioremediation.

Introduction

A large amount of sludge discharged from the pulp and paper industry contains hazardous toxic pollutants along with organic and inorganic compounds (Abedinzadeh et al. 2018). Paper is one of the most widely utilized commodities in our everyday lives, appearing in a variety of formats such as office work, newspapers, school, and so on. Researchers have designed several approaches that can be used in the pulp and paper industry to minimize hazardous elements in sludge and effluent using plants and microorganisms (Sharma et al. 2021). Worldwide, the paper industry's release of toxic organics with liquid and solid waste has become a serious concern (Sonkar et al. 2021). In 2006 and 2007, the paper industry consumed six percent and five percent of global industrial energy consumption, respectively (Posch et al., 2015). This industry uses a high amount of freshwater, between 60-230 m³ per tonne of paper manufacturing, resulting in a considerable quantity of sludge becoming produced (Buyukkamaci and Koken 2010). In various stages of the paper-making process in the paper and paper industry, sludge has identified 240-250 chemicals (Ram et al. 2020). In the bleaching process, up to 85 % of the total sludge volume is produced. The immense amount of fresh water is used in a different stage of papermaking in the industry (Sharma and Rath 2021). The toxic constituents in this sludge must be treated before being discharged into surface water bodies. More hazardous effluent and sludge are produced by many small-scale industries (Dixit et al. 2020). Before final discharge, the toxicity of the sludge surroundings must be evaluated. Industrial sludge is the primary source of environmental pollution globally due to the release of metals and metalloid contents in water, soil, and air and causes mutagenic and androgenic effects on plants and fishes (Ngo et al. 2020; Sharma et al. 2020).

However, due to their high cost, the implementation of several approaches in the industry is not economically viable to a considerable extent (Sharma and Singh 2021). However, due to their high price,

the performance of several methods in the industry is not financially feasible to a substantial extent. The main product of the pulp and paper industry is sludge, as it ranks third among liquid industries (Ginni et al. 2014). These pollutants are degraded by microbes into less harmful molecules, reducing the requirement for chemicals throughout the process of recovery. Even before the pulp and paper industry's sludge treatment with microbes improved methane yield, successful final destruction of adsorbable organic halides (AOX) was reported (Lin et al. 2017; Mehmood et al. 2019). Industrial sludge treatment methods like ozonation, hydrogen peroxide, UV treatment, and the photo-Fenton process are also promoted to treat sludge from the pulp and paper industry and extract color and odor (Ajmi et al. 2018). Bacteria such as *Clostridium thermocellum* may also produce pulp and paper sludge methane (An et al. 2020). Microorganisms can thus perform efficiently and with high returns. As compared to single-phase hydrogen fermentation and mesophilic methane generation, two-phase hydrogen fermentation and mesophilic methane generation have a 50 % increase in energy acquiescence. The microbial community is highly affected by polluted sludge, and it is enhanced the remediation of several organic and inorganic pollution parameters (Kim et al. 2020; Sharma et al. 2021a).

The use of enzymes produced from microbes will minimize sludge toxicity and enhance the property of the pulp for improved application (Valls et al. 2010). Microorganism-applied sludge treatment is based on its biosynthetic enzymes. Different system biology techniques are being used to change the metabolic process of microbes to fasten sludge degradation. Various microorganisms were tested to break down harmful components in industrial waste (Sharma et al. 2021b, c). Biological treatment is a unique method of reducing the toxicity of sludge and making it safe for reuse (Gupta et al. 2019). Metagenomic research is an effective strategy for retailing microbial communities like those that work within sludge treatment plants (Rosso et al. 2019). More than 99% of microbes are complex but cannot be purely cultivated in the field of microbiology, implying that our understanding of microorganisms is essentially limited to far less than 1% of microorganisms. A cultivation-independent technique, e. g metagenomics, will become a competent method for analyzing the capacity of certain microbes for refining with the rapid progress of high-throughput sequencing (Kumar et al. 2020). Metagenomic microbial sequencing is a capable alternative to rRNA sequencing to analyze the complex structure of the microbial community and enable the intrinsic composition and utilitarian potential of a microbial community to be screened (Jadeja et al. 2014; Lou et al. 2019). Metagenomics of sludge samples provides valuable insights concerning factors related to human health, such as the spread of infections and genes for antibiotic resistance (Schneider, 2020). Sludge treatment plants are an important modern facility that uses a diverse microbial community to break down organic materials and remove hazardous microorganisms from the ecosystem.

Metagenomic analysis can be performed on PCR-amplified 16S rRNA fragments or all DNA isolated from biological samples (shotgun MGA). For every case, nucleotide sequences decide millions of DNA fragments. Statistical techniques are used to categorize and quantify species as long as all species (or genes) are included in vast and expanding databases (Gilbert and DuPont 2011; Vanwonterghem et al. 2014). MGA is gaining traction in the selected industry as a reliable method of getting knowledge into processes of vital importance to the sludge community (Jiang et al. 2016). In 2011, the Earth Microbiome

Project was begun, along with the development of low-cost, next-generation high-performance sequencing tools, to uncover the vast, untapped microbial genetic resources found in soil, saltwater, freshwater, the atmosphere, and other ecosystems (Guo and Zhang 2013). Activated sludge (AS) in environmental samples is almost entirely made up of bacterial cells or components, including extracellular polymeric polymers (EPS) (Liu and Fang 2003). One gram of AS dry mass is typically produced by $1\text{-}10^{10}\text{-}10^{12}$ bacterial cells (about $1\text{-}10^{10}$ cells per milliliter of working activated sludge) (Nielsen and Nielsen 2001). The microbial density in soil samples is over 100 times higher than this value. For its abundance, biomass was not an issue, and only a few hundred microliters to several milliliters of sludge are required for DNA extraction. Molecular and sequencing approaches offer qualitative and quantitative insights into the environment of polluted water (McCall and Xagorarakis, 2019). In environmental systems, metagenomics facilitates the sequencing of a broad set of microbes which would otherwise prove time-consuming using conventional laboratory techniques. In ecological microbial community investigations, standardizing of DNA extraction is a basic concern of accuracy and comparability. Metagenomics is a diverse strategy for identifying the microbial environment and has made considerable progress about the awareness and significance of non-cultural microbes between the study community and industrial application. An effective technique to classify the source of metabolites from non-cultivable microbes is functional metagenomics. Functional metagenomics, moreover, still faces many problems yet to be addressed since this technology is moving via stages of development. Enhancements in metagenomic technologies were valuable.

The advent of several metagenomic divisions demonstrates the development of the techniques and quick and financially viable sequencing evaluation. This will also emphasize the development of a potential field of study in genetic engineering, molecular science, microbiology, food science, and pharmaceuticals. As a result, recovering beneficial metabolites advances customer demand and economic viability, a feasible final product that can still be manufactured on an industrial scale, and prospective problems in another research area. In recent years, understanding the difficulties of microbial communities, the biological system, and their interactions with external variables has necessitated an effective combination of multiple techniques based on metagenomics. To summarise, modern 'metagenomics' technologies may be a competent approach for classifying microbial communities, making them more intelligible, informative, multi-purpose, and financially viable. This study provides a summary of the possibility for metagenomics technologies to be improved to build a new microbial community from uncultured microorganisms.

1. Materials And Methods

1.1. Site detail and sample collection

Star Pulp and Paper Limited, Saharanpur, India, Latitude: $29^{\circ} 56' 10.89''$ N, Longitude: $77^{\circ} 34' 13.51''$ E, supplied the sludge sample. The industry-based uses mostly agrofuel coal and lignite and produces 560 tonnes of wastewater per day. Agro fuel includes wild grass, mustered straw, bagasse, rice husk, paddy, wood chips, and cane trash. The evaluation took place at the Star pulp and paper plant in Saharanpur,

Uttar Pradesh, which is one of the country's oldest and largest paper mills. Finding demonstrates that sludge and effluent generated by paper industries contain several harmful chemicals, like metals, lignin, chlorides, ions, and suspended solids, that can be incredibly hazardous in rivers and lakes (Sharma et al. 2020a). Sludge from the pulp and paper industry was collected into 20-kilogram sterile plastic tanks that were sterilized (Tarsons Production Pvt. Ltd., USA).

1.2. DNA isolation and purification

Following the extraction of DNA from the sludge sample, the concentration of DNA was determined using a "Qubit Fluorimeter (V.3.0). The concentration of extracted community DNA was evaluated at 260/280 nm and 260/230 nm absorption ratios using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). V3 Forward primer CCTACGGGNNBGCASCAG and V4 Reverse primer GACTACNVGGGTATCTAATCC were used to amplify the V3-V4 region of 16S rRNA. On a 2% agarose gel, the amplified product was examined, and gel purification was performed to remove non-specific amplifications". The NEBNext Ultra DNA library preparation kit was used to prepare 5ng of amplified product for library preparation. The Agilent 2200 TapeStation was used to quantify the library and estimate its quality (Kumbhare et al. 2015). The Illumina HiSeq 2500 platform was used to sequence the prepared library. The 16S rRNA gene's V3-V4 region was amplified using a polymerase chain reaction (PCR) reaction step, denaturation, and a final extension, and the PCR amplicon was purified with AMPure XP beads (Agencourt Bioscience, USA).

1.3. Fast and base quality check

The raw reads obtained from the Illumina sequencing platform after demultiplexing were "subjected to the FastQC program (latest version.0.11.8) to check the quality of the reads with default parameters. The base quality (Phred Score; Q), base composition, guanine-cytosine (GC) content, ambiguous bases (other than adenine [A], thymine [T], guanine [G], cytosine [C]), and adapter dimers were thoroughly checked before the Bioinformatics analysis". Moreover, the base quality of each cycle, the x-axis represents the sequencing cycle, and the y-axis represents the percentage of total reads.

1.4. 16s rRNA amplicon-based Illumina library preparation

The Illumina MiSeq network protocols were used to create the 16S rRNA gene library (<https://sapac.illumina.com/systems/sequencing-platforms.html>). Operational Taxonomic Units (OTUs) and taxonomic classification were determined using pre-processed consensus V3-V4 sequences. The Uclust algorithm (similarity cutoff = 0.97) in QIIME software was used to pool pre-processed reads from all samples and cluster them into OTUs based on their sequence similarity. Furthermore, by mapping each representative sequence to the SILVA OTUs database, taxonomy categorization was done using the RDP classifier. Using the PyNAST tool, representative sequences from each clustered OTU were selected and aligned against the SILVA core set of sequences.

1.5. Statical analysis

The bacterial community was studied using V3-V4 16S rRNA sequencing data generated by the Illumina MiSeq framework. The Quantitative Insights in Microbial Ecology (QIIME) was utilized to cluster high-quality readings into OTUs at the 97 % identification stage (Caporaso et al., 2010). The descriptive sequences of each clustered OTU were chosen and matched against the SILVA core set of sequences using the PyNAST program. Furthermore, each descriptive sequence was mapped to the SILVA OTUs database using the RDP classifier for taxonomy categorization. OTUs at the phylum, class, order, family, genus, and species levels are used to create the maximum probability figure.

2. Results And Discussion

2.1. Base quality and GC- contents

Many metabolic pathways are associated with either their use of nucleic acid or nucleotides or regulated by such enzymes (Kilstrup et al. 2005). The base composition of sludge showed adenine (A- 22.81 %), guanine (G-25.49 %), thionine (T-22.80 %), and cytosine (C-26.09 %), respectively. Relevant parameters are the composition of nucleic acids in the sequence read from sludge. In different environments, most bacteria are capable of developing nucleic acids under different conditions. The base quality check showed the x-axis is the sequencing period. The total nucleic acids sequencing cycle is determined by the y-axis, and the proportion of total reads is represented by the y-axis. These diagrams indicate the quality of the left and right ends of the sample's paired-end read sequences. As shown, more than 80% of the total reads have a Phred score of greater than 30 ($>Q30$; error-probability ≥ 0.001). The left and right ends of paired-end read sequences' base composition are calculated. Although the target sequence is that of the composition bias of the V3-V4 zone sequence in the sludge sample. Microbes can use nucleotides as purine or pyrimidine sources, but intracellular nucleosidases must dephosphorylate these before entering the cell. The average GC content distribution of sequenced reads from sludge samples is between 30 and 60%. The average GC content in the sequence is represented on the x-axis, while the proportion of sequences is represented on the y-axis. The quality of the genome assemblies depends on the sequencing technologies used and on the purposes for which the sequence was developed (Smits 2019). Protein genes, functional RNA genes, and spacers, for example, have a considerable linear association with the G+C composition of their genomic DNA, as seen in Fig. 1.

2.2. Assessment of microbial diversity

Members of various taxonomic, biochemical groups such as nitrification, N_2 -fixation, sulfur oxidation, denitrification, and physiological groups such as heterotrophic, aerobic, anaerobic, phototrophic, groups include bacterial sludge communities, which have functional advantages for water detoxification (Numberger et al. 2019). The ability to profile the makeup of ambient and host-associated microbial diversity has greatly enhanced owing to metagenomics (Milanese et al. 2019). Toxin removal from industrial waste is a major problem for industry (Mandeep 2020). Because of the potential environmental benefits of utilizing photosynthetic organisms for bioremediation, microalgae application in sludge treatment is gaining traction (Sforza et al. 2020). In 159037 reads, a total of 9596 OTUs were discovered.

7993 OTUs with less than 5 reads were deleted from the total of 9596 OTUs, and 1603 OTUs were chosen for further study. The relative abundance plot at class, family, phylum, genus, order, and species level is based on OTUs. This can be noticed that over 80% of the total reads have a Phred score of 30 or higher ($>Q30$; error-probability ≥ 0.001). Phred quality score distribution (%) were Q0-Q10 (2.81), Q10-Q20 (6.34), Q20-Q30 (4.43) $\geq Q30$ (86.42). Co-occurrence network analysis can be used to evaluate potential links between microbial communities and assess the structures of complex microbial communities across spatiotemporal gradients.

2.3. Microbial community at class, family, genus, and species level

Microbes found in sludge treatment are important for the purification of sludge to safeguard the health and environmental health (Wu et al., 2019). Results revealed that 16s rRNA amplicon-based Illumina sequencing was conducted to identify the bacterial diversity and richness and compared to the similarity or difference of microbial structures at different taxonomic levels, identify specific populations, and evaluate sludge functional genera. This is universally accepted that the efficiency of different biological treatment processes depends primarily on the behavior and communication of microbes, and the efficacy of removal of pollutants is usually positively linked to the microbiota (Zhang et al. 2016).

The properly paired-end reads with Phred score quality ($Q>20$) were considered for V3-V4 consensus generation. The primer trimmed; high-quality paired-end reads were pair-wisely allowed to merge/stitch to get the V3-V4 amplicon consensus FASTA sequences. In pulp and paper industry sludge, the top ten represented at the phylum level were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Spirochetes*, *Tenericutes*, *Euryarchaeota*, *Chloroflexi*, *Verrucomicrobia*, *Actinobacteria*, and including unknown microbial community (Fig. 2a). Chemical oxygen demand (COD), total nitrogen (TN), and total phosphorus (TP) are all removed efficiently by *Proteobacteria* (Wu et al. 2016). Moreover, an unknown bacteria phylum was also found among the top bacterial families. *Proteobacteria* are found in a variety of habitats and play an important role in the breakdown of chemical molecules. Salinity has been earlier mentioned as a significant factor impacting *Proteobacteria* proliferation. *Proteobacteria* gates have been discovered to metabolize soluble organic substrates (Shao et al. 2013). While the class-level top genera identified were *Clostridia*, *Bacteriodia*, *Gammaproteobacteria*, *Spirochetia*, *Deltaproteobacteria*, *Erysipelotrichia*, *Mollicutes*, Unculture microbes, and *Anaerolines* (Fig. 2b). In addition, among the top bacterial families, an undiscovered bacteria class was identified. *Gammaproteobacteria* were prevalent in a range of habitats and were crucial in the degradation and deionization of pollutants.

Moreover, at the family level, dominant species were *Rumminococcaceae*, *Anaerolineaceae*, *Spirochaetaceae*, *Prolixibacteraceae*, *Clostridia-vadinBB60-group*, *Paludibacteraceae*, and *Acidaminocaccaceae* (Fig. 3a). In addition, an unidentified bacteria family was discovered among the top microbial species. The dominant order level was *Spirochateales*, *Bacteroidales*, *Pseudomonadales*, *Betaproteobacteria*, *Selenmonadales*, *Erysipelotrichales*, *Synergistales*, and *Anaerolineales* (Fig. 3b). In addition, among the top bacterial families, an unknown bacteria order was discovered. Bacteroidales

members have colonized a variety of ecological settings, demonstrating a wide range of biological roles. On the other hand, lignocellulosic materials are abundant organic and inorganic materials that can be used as the sole source of carbon and nitrogen for bacteria to flourish at pulp and paper industry polluted locations (Womersley 2006). Bacteroidales have also been discovered to have no link to eutrophication, while Actinobacteria abundance is high in low-eutrophication locations (Xue et al. 2018). *Beta-proteobacteria* is most known for its capacity to aerobically oxidize sulfur compounds. *Beta-proteobacteria* often plays an important role in the sequestration of nutrients and the elimination of other sludge pollutants. *Pseudomonas* has the remarkable ability to break down many polymers that are difficult to decompose by any other microbial communities (Shahid et al. 2020). Bacterial biofilm plays a crucial role in removing organic, inorganic contaminants, and metals from various sludge sources. The extracellular DNA also enhances the biofilms' structural stability. For instance, *Pseudomonas aeruginosa* has a large amount of DNA to maintain biofilm integrity (Rice et al. 2007).

The profiling of dominant genus and species level *Rumminococcaceae*-UCG-010, *Erysipelotrichales*, *Rumminococcaceae*-NK4A214-group, *Treponema*-2, *Desulfovibrio*, *Anaerolinea*, *Pseudomonas*, *Verrucomicrobia*-bacteria-ADurb.Bin070, *Selenivibrio*-woodroffi, and *Inostemma*sp-AD-2014 (Fig. 4a-c). *Erysipelotrichales* was detected in pulp and paper industry sludge, it was the second-highest of all the species that were found. *Verrucomicrobia* is inadequately defined and cultured in the laboratory, which contributes to its growth characteristics that result in dormancy (Bergmann et al. 2011). As a result, these findings suggested that the microorganisms studied may have a wide range of metabolic capacities, including the ability to use a variety of sugars and organic molecules as a sole nitrogen and carbon source. As a result, this ability establishes a niche for these growing bacterial populations, resulting in in situ harmful pollutant bioremediation. In the detoxification of heavy metals, *Pseudomonas* plays a crucial role (Wang et al. 2018). In our co-occurrence network, these microbes also perform an important ecological role.

2.4. α -diversity and rarefaction curves

We used the Illumina sequencing method in this work to investigate the microbial community in sludge samples collected from regions at low altitudes. For sludge samples, the raw sequence reads 334091, and the sequence length is 250 at the sequencing level. The alpha diversity indices differed significantly from sludge treatment systems (Fang et al. 2018). Results showed the highest OTUs in 9596. Shannon's diversity index showed significant microbial diversity of pulp and paper industry sludge samples ($t = 3.325$, $p < .05$). This section calculates Shannon, Chao1, and observed species metrics to assess microbial diversity in sludge samples shown in Fig. 5, 6, and 7. The chao1 metric evaluates species richness, whereas the Shannon metric accounts for both richness and evenness when estimating observed OTU abundances. The count of unique OTUs found in the sludge is the observed species metric. To see if sequencing depth is adequate to accurately capture the total bacterial diversity in the ambient samples, we created rarefaction curves. The Shannon index and Chao1 richness estimator, on the other hand, report that microbial communities are extremely rich and varied in rhizospheric zones. Within these

phyla, classes, orders, families, genera, and species that evolved, the phyla, classes, orders, families, genera, and species were subsequently classified.

The composition and diversity of bacterial communities in environmental samples can be studied using next-generation sequencing techniques. They discovered significant differences in bacterial community structure across pulp and paper industry sludge using Illumina (MiSeq) sequencing of the V3-V4 hypervariable region and bacterial 16S rRNA gene analysis. We did rarefaction sampling on our alphabets sludge samples and obtained the Chao1 species richness estimation and Shannon diversity indices. Sludge bacterial communities appeared to be far more diverse according to the Chao1 and Shannon-D indices. Environmental variables, such as soil properties, levels of pollutants, plant root exudates, and conditions, had a profound impact on microbial diversity (Erguven et al. 2016). The overall nature and diversity of the microbial communities in the substrate all determine how much pollutants can be removed. When the microbial population in the lake water decreases, the nutrient cycles shift, and a cyclic process is produced. The structure of microphytic communities is influenced by a variety of environmental factors (Shang et al. 2020). Several different indexes are used in ecological investigations. Though the Shannon-Wiener Index is the most widely utilized due to its smaller sample size and higher sensitivity, it is worth noting that the index is specifically beneficial in characterizing the pollution and trophic condition of aquatic species. The increased diversity of species was also highly connected with the temperature of the environment, which was reflected in the richness of species.

Conclusion

The study showed that microbial diversity has a positive correlation with the nutrients present in pulp and paper industry sludge after secondary treatment. The bacterial community was dominated by *Proteobacteria*, *Pseudomonadales*, *Betaproteobacteria*, *Selenmonadales*, *Bacteroidetes*, *Actinobacteria*, and a large number of unknown microorganisms, while it is unknown exactly how important this function is, it might have a critical impact on ecosystem development and maintenance. Species diversity is generally used in the assessment of microbial ecology during bioremediation. The microbial community's in-depth identification will help understand its metabolic mechanisms, which can be valuable for cleaning certain polluted water. Several microorganisms are probably involved in the biodegradation and/or biotransformation of organic contaminants detected in sludge. This research offered evidence on the relationship between microbial networks and diverse sludge pollutants. The focus of future research should be on determining the specific role of indigenous microbes in a contaminated environment.

Declarations

CRedit authorship contribution statement

Pooja Sharma: Experiment designed, writing - original draft, Writing - review editing, sample collection, and analysis of data.

Surendra Pratap Singh: Data analysis, Visualization, reviewing, and editing manuscript. All authors have read and agreed to the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

Figure 1

Molecular size of extracted DNA by qubit concentration methods as visualized by agarose gel electrophoresis.

Figure 2

Phylum and class level of relative abundance of microbes in industrial sludge (a). Relative abundance at phylum level (OTUs) and (b). class level (OTUs).

Figure 3

Family and order level of relative abundance of microbes in industrial sludge (a). Relative abundance at family (OTUs) and (b). order level (OTUs).

Figure 4

Genus and species level of relative abundance of microbes in industrial sludge (a). Relative abundance at genus (OTUs) and (b). species level (OTUs).

Figure 5

Shannon curve obtained for the sludge sample.

Figure 6

Chao1 curve obtained for the sludge sample.

Figure 7

Observed species curve obtained for the sludge sample.